

**Preliminary Amendment**

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Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

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**Remarks**

Prior to taking up the above-identified application for examination, the Examiner is asked to enter the above amendment. These amendments to the specification simply correct typographical errors and add no new matter to the specification.

The first amendment made on page 2 was made to eliminate a reference to an author that does not appear in the book cited. The editor, book title, and publisher information were correctly cited, and from this information the book can be easily found. The latter two amendments made to page 2 correct the spelling of the author's name.

The amendment on page 25 was made to correct the volume number of the cited document. The author, journal title, page numbers and year of publication were correctly cited, and a search of the literature by author, journal title and year, taken in the context of the passage in the specification where the citation occurs, would enable one skilled in the art to determine the correct citation of the document.

The amendments made on pages 30 and 32 were made to complete the citation and distinguish the journal title from other similar works.

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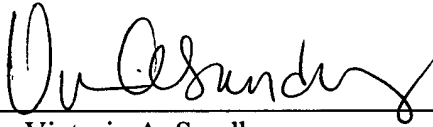
The Examiner is invited to contact Applicants' Representatives, at the below-listed telephone number, if there are any questions regarding the above preliminary amendment, or if prosecution of this application may be assisted thereby.

Respectfully submitted,

Przybyla et al.

By their Representatives,  
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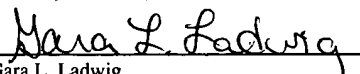
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Name: Gara L. Ladwig

APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS INCLUDING NOTATIONS TO  
INDICATE CHANGES MADE

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Docket No. 235.0004 0101

Amendments to the following are indicated by underlining what has been added and bracketing what has been deleted. Additionally, all amendments have been shaded.

Page 2, line 3 to line 28

Some of the strategies employed to overcome the problems of protein stability and solubility in *E. coli* include the use of fusion partners such as maltose binding protein (31 kD) ([P. Riggs, in] Ausebel, F.M. et al. (Eds) *Current Protocols in Molecular Biology*, Greene Associates/Wiley Interscience, N.Y. (1990)), thioredoxin (U.S. Pat. No. 5,646,016, issued Jul. 8, 1997; U.S. Pat. No. 5,270,181, issued Dec. 14, 1993; U.S. Pat. No. 5,292,646, issued Mar. 8, 1994) and glutathione-S-transferase (28kD) (D. Smith et al., *Gene* 67: 31-40 (1988); U.S. Pat. No. 5,654,176); and the use of protease deficient strains of *E. coli* (Bibi et al., *Proc. Nat'l. Acad. Sci. (USA)* 90 :9209 (1993); D. Alexander et al., *Protein Exp. Purif.*, 3:204 (1992)). The importance of the cellular redox environment as a factor affecting folding and solubility of foreign proteins has been demonstrated through the use of the redox-active protein thioredoxin (12kD) as a fusion partner in expression systems (E. [Lavallie] LaVallie et al., *Biotechnology* 11:[18] 187 (1993)) and through the synthesis of proteins in thioredoxin reductase (trx-) negative strains of *E. coli* (A. [Darman] Derman et al., *Science* 262:1744 (1993)). These fusion systems have proven very useful, but the fusion products are sometimes difficult to follow during purification and there is still no assurance that any given protein will fold properly and/or become or remain soluble in any of the fusion systems in current use. Moreover, although the fusion partners maltose binding protein, glutathione-S-transferase and thioredoxin are typically derived from bacteria or protozoa, the existence of closely related mammalian and avian analogues of these fusion partners makes them unsuitable for use as anchor proteins for haptens in antibody production or in vaccines. Thus, continued development of new protein expression systems based on recombinant protein fusions with a stable carrier is necessary to advance the art of recombinant protein production.

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Page 25 line 27 to page 26 line 10

Rubredoxins from numerous different organisms have been isolated, and the amino acid sequences of various rubredoxins and the genes encoding various rubredoxins have been published. In this experiment the gene encoding rubredoxin from *D. vulgaris* St. Hildenborough was used (see Fig. 1; also Bruschi et al., *Adv. Exp. Med. Biol.* 74:57-67 (1976); Voordouw, *Gene* 69: 67: 75-83 (1988)). The gene was amplified by polymerase chain reaction (PCR) from genomic DNA isolated from *D. vulgaris* using two primers and cloned into the expression vector pET24a (Novagen, Wisconsin) at the *Nde* I and *Bam*HI site. The pET-24a expression system utilizes the bacteriophage T7 promoter that serves as a binding site for T7 RNA polymerase and was incorporated into the chromosomal DNA of *E. coli* strain BL21 (DE3) (Novagen). T7 RNA polymerase is synthesized only upon the addition of isopropyl  $\beta$ -D-thiogalactoside (IPTG) to growing cultures since the gene for the T7 polymerase has been spliced into the chromosomal DNA of the *E. coli* host. The pET-24a plasmid also contains the gene for kanamycin resistance for selection of plasmid-containing colonies.

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Page 30, line 6 to line 24

Previous attempts to synthesize recombinant amyloid peptide in *E. coli* have resulted in the formation of inclusion bodies that required the use of guanidine thiocyanate for solubilization (B. Boyes et al., *J. Chromatog* 4, 691:337 (1995); Gardella et al., *Biochem. J.* 294:667-674 (1993)). A method for synthesizing this peptide as a recombinant fusion protein occurring in inclusion bodies was previously developed at Hoffman-La Roche (Döbeli, et al., *Biotechnology* 13:988-993 (1995)), but processing of their fusion to form pure monomeric A $\beta$ <sub>1-42</sub> is tedious in that it involves binding the fusion protein to a reverse-phase column followed by cyanogen bromide (CNBr) cleavage to remove the peptide from the fusion. Analysis of peptide purified with this method revealed formylation and carbamylation of the peptide as well as oxidation of Met-35. These alterations presumably occur as a result of CNBr cleavage of the peptide; Met-35 must be reduced by dimethylsulfoxide (DMSO) treatment in concentrated hydrochloric acid (HCl) before use. In this example, amyloid peptides were synthesized as fusions with rubredoxin in the hope of circumventing the difficulties of synthesizing homogeneous and consistently pure, monomeric peptides using existing methods. Recombinant synthesis as fusion proteins also allows more economical production of labeled peptides for use in continuing medical research efforts.

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Page 32, line 15 to page 33, line 23

*Purification of  $\beta$ -amyloid 1-42 and 1-40*

Following cleavage, the property of the  $A\beta_{1-42}$  and  $A\beta_{1-40}$  peptides to form sedimentable aggregates was used to concentrate and purify the peptide away from most of the rubredoxin moiety. But non-specific cleavage of both amyloid fusion proteins that occurs after Arginine-5 generated an additional peptide fragment that had to be separated from the intact peptides. The propensity of  $\beta$ -amyloid 1-42 to form aggregates and insoluble fibers poses a major problem in purifying this peptide (D. Burdick et al., *J. Biol. Chem.* 267:546 (1992), P. Sweeney et al., *Anal. Biochem.* 212:179 (1993)). Normal reverse phase chromatography is not a suitable method for purification. High temperature reverse phase chromatography using a Zorbax Stable Bond C18 column (McMod, PA) (B. Boyes et al., *J. Chromatog.* 691:337 (1995)) at pH 2.5 (0.05%TFA) was thus attempted. Temperatures in the range of 80-85°C resulted in good resolution between  $\beta$ -amyloid 1-42 and the various contaminating peaks. The  $\beta$ -amyloid 1-42 peptide isolated by this protocol was found to be pure as judged by mass spectrometry and was free of chemical modifications. However, this method poses a problem in that the temperatures used are very close to the boiling point of acetonitrile and further, heating a scale up preparatory column is a long and expensive proposition. Moreover, it is difficult to work at a pH above about pH 6 with silica based resins since at high temperatures silica tends to degrade at a pH above 5.

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### In the Claims

For convenience, all pending claims are shown below.

45. (New) A recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

**46. (New) The recombinant polynucleotide of claim 45 wherein the N-terminal rubredoxin constituent is cleavably linked to the C-terminal fused polypeptide.**

47. (New) The recombinant polynucleotide of claim 45 wherein the rubredoxin fusion protein further comprises an intervening spacer region positioned between the N-terminal rubredoxin constituent and the C-terminal fused polypeptide.

48. (New) The recombinant polynucleotide of claim 45 wherein the intervening spacer region comprises at least one component selected from the group consisting of a proteolytic cleavage site and an affinity purification sequence.

49. (New) A recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

**50. (New) An expression vector comprising:**

a nucleotide sequence encoding rubredoxin or a biologically active analogue, fragment, or modification thereof;

an intervening nucleotide sequence encoding a spacer region; and

a multiple cloning region comprising at least one restriction endonuclease recognition

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site.

51. (New) The expression vector of claim 50 wherein the intervening nucleotide sequence comprises all or a portion of the multiple cloning region.

52. (New) The expression vector of claim 51 which is pRUBEX3, wherein pRUBEX3 comprises a nucleotide sequence encoding an affinity tag having at least one amino acid sequence selected from the group consisting of His-His-His-His-His-His (SEQ ID NO:4) and His-Gly-Leu-His (SEQ ID NO:7).

53. (New) The expression vector of claim 50 wherein the intervening nucleotide sequence encodes at least one of a proteolytic cleavage site and an affinity purification sequence.

54. (New) An expression vector comprising a promoter operably linked to a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

55. (New) An expression vector comprising a promoter operably linked to a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

56. (New) A host cell transformed with an expression vector comprising a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.



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57. (New) The host cell of claim 56 which is a bacterial cell.

58. (New) A host cell transformed with an expression vector comprising a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

59. (New) The host cell of claim 58 which is a bacterial cell.

60. (New) A method for making a rubredoxin fusion protein comprising:

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide; and

(b) expressing the fusion protein in the host cell, wherein the fusion protein binds a divalent cation and is chromogenic.

61. (New) The method of claim 60 wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound, wherein the fusion protein expressed in the host cell in step (b) is isotopically labeled.

62. (New) The method of claim 61 wherein the host cell is an amino acid auxotroph.

63. (New) The method of claim 61 wherein the fused polypeptide is isotopically labeled with at least one of  $^{35}\text{S}$ ,  $^{13}\text{C}$ , or  $^{15}\text{N}$ .

64. (New) The method of claim 60 wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

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65. (New) The method of claim 60 further comprising (c) removing the fusion protein from the host cell.

66. (New) The method of claim 65 further comprising (d) purifying the fusion protein.

67. (New) The method of claim 66 wherein step (d) comprises visually tracking the location of the fusion protein.

68. (New) The method of claim 66 wherein step (d) comprising purifying the fusion protein using reverse phase chromatography at temperatures between about 45°C and about 65°C.

69. (New) The method of claim 66 further comprising (e) cleaving the fusion protein to yield the rubredoxin constituent and the polypeptide.

70. (New) The method of claim 69 further comprising (f) purifying the polypeptide using reverse phase chromatography at temperatures between about 45°C and about 65°C.

71. (New) A method for making a polypeptide which, when not fused to a rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system, the method comprising:

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system; and

(b) expressing the fusion protein in the host cell.

72. (New) The method of claim 71 wherein the host cell contains or is supplied with at least one

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isotopically labeled amino acid or precursor compound, wherein the fused protein expressed in the host cell in step (b) is isotopically labeled.

73. (New) The method of claim 72 wherein the host cell is an amino acid auxotroph.

74. (New) The method of claim 72 wherein the fused protein is isotopically labeled with at least one of  $^{35}\text{S}$ ,  $^{13}\text{C}$ , or  $^{15}\text{N}$ .

75. (New) The method of claim 72 wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

76. (New) The method of claim 71 further comprising (c) removing the fusion protein from the host cell.

77. (New) The method of claim 71 further comprising (d) purifying the fusion protein.

78. (New) The method of claim 71 wherein step (d) comprises visually tracking the location of the fusion protein.

79. (New) The method of claim 71 wherein the fusion protein is purified using reverse phase chromatography at temperatures between about 45°C and about 65°C.

80. (New) The method of claim 77 further comprising (e) cleaving the fusion protein to yield the rubredoxin constituent and the polypeptide.

81. (New) The method of claim 80 further comprising (f) purifying the polypeptide using reverse phase chromatography at temperatures between about 45°C and about 65°C.

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82. (New) A method for making a rubredoxin- $\beta$ -amyloid fusion protein comprising:

- (a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused  $\beta$ -amyloid peptide, wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound; and
- (b) expressing a rubredoxin- $\beta$ -amyloid fusion protein in the host cell wherein the fused  $\beta$ -amyloid peptide is uniformly isotopically labeled.

83. (New) The method of claim 82 wherein the rubredoxin- $\beta$ -amyloid fusion protein is uniformly labeled with at least one of  $^{35}\text{S}$  and  $^{15}\text{N}$ .

84. (New) The method of claim 82 further comprising (c) removing the rubredoxin- $\beta$ -amyloid fusion protein from the host cell and (d) purifying the rubredoxin- $\beta$ -amyloid fusion protein using reverse phase chromatography at temperatures between about 45°C and about 65°C.

85. (New) The method of claim 82 further comprising cleaving the rubredoxin- $\beta$ -amyloid fusion protein to yield the rubredoxin constituent and the  $\beta$ -amyloid peptide.

86. (New) The method of claim 85 further comprising purifying the  $\beta$ -amyloid peptide using reverse phase chromatography at temperatures between about 45°C and about 65°C.

87. (New) A method for making a rubredoxin- $\beta$ -amyloid fusion protein comprising:

- (a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused  $\beta$ -amyloid peptide, wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound selected from the group consisting of  $^{35}\text{S}$ -methionine and an  $^{15}\text{N}$ -labeled precursor compound; and

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(b) expressing a rubredoxin- $\beta$ -amyloid fusion protein in the host cell wherein the fused  $\beta$ -amyloid peptide is uniformly labeled with at least one of  $^{35}\text{S}$  and  $^{15}\text{N}$ .

88. (New) A rubredoxin fusion protein comprising a chromogenic N-terminal rubredoxin constituent and a C-terminal fused polypeptide.

89. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

90. (New) The rubredoxin fusion protein of claim 89 which is soluble when overexpressed in a host cell.

91. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide selected from the group consisting of an amyloid peptide, leptin, proinsulin, trypsin inhibitor, the extracellular domain of luteinizing hormone receptor, and a biologically active fragment, modification or analogue of any of the preceding polypeptides.

92. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

93. (New) The rubredoxin fusion protein of claim 92 comprising a detectable label.

94. (New) The rubredoxin fusion protein of claim 93 wherein the detectable label is a mass isotope or a radioisotope.

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95. (New) The rubredoxin fusion protein of claim 93 wherein the detectable label is selected from the group consisting of  $^{35}\text{S}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ .

96. (New) The rubredoxin fusion protein of claim 95 which is uniformly labeled with the detectable label.

97. (New) The rubredoxin fusion protein of claim 95 which is uniformly labeled with both  $^{35}\text{S}$  and  $^{15}\text{N}$ .

98. (New) The rubredoxin fusion protein of claim 92 wherein the N-terminal rubredoxin constituent is cleavably linked to the C-terminal fused polypeptide.

99. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -labeled  $\beta$ -amyloid peptide.

100. (New) The rubredoxin fusion protein of claim 99 wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -methionine-labeled  $\beta$ -amyloid peptide.

101. (New) The rubredoxin fusion protein of claim 99 wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -labeled 1-42  $\beta$ -amyloid peptide.

102. (New) The rubredoxin fusion protein of claim 99 wherein  $\beta$ -amyloid peptide is uniformly labeled with  $^{35}\text{S}$ .

103. (New) The rubredoxin fusion protein of claim 99 wherein  $\beta$ -amyloid peptide is uniformly labeled with  $^{35}\text{S}$  and  $^{15}\text{N}$ .

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104. (New) An  $^{35}\text{S}$ -labeled  $\beta$ -amyloid peptide.

105. (New) An  $^{35}\text{S}$ -methionine-labeled  $\beta$ -amyloid peptide.

106. (New) The  $^{35}\text{S}$ -methionine labeled  $\beta$ -amyloid peptide selected from the group consisting of an  $^{35}\text{S}$ -methionine labeled 1-42  $\beta$ -amyloid peptide and an  $^{35}\text{S}$ -methionine labeled 1-40  $\beta$ -amyloid peptide.

107. (New) A  $\beta$ -amyloid peptide that is uniformly labeled with  $^{35}\text{S}$  and  $^{15}\text{N}$ .

108. (New) A method for making an antibody comprising eliciting in a host cell an immune response to an antigen comprising a rubredoxin fusion protein comprising a N-terminal rubredoxin constituent and a C-terminal fused polypeptide to yield antibodies to the fused polypeptide.

109. (New) The method of claim 108 wherein the antibody is a polyclonal antibody.

110. (New) The method of claim 108 wherein the antibody is a monoclonal antibody.

111. (New) The method of claim 108 where the antibody is not cross-reactive with rubredoxin.

112. (New) A vaccine comprising:

at least one component selected from the group consisting of:

(a) a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide;

(b) a polynucleotide comprising a nucleotide sequence encoding said rubredoxin fusion protein; and

a pharmaceutically acceptable carrier.

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**113. (New) The vaccine of claim 112 wherein the N-terminal rubredoxin constituent is directly linked to the C-terminal fused polypeptide.**

**114. (New) The vaccine of claim 112 further comprising an adjuvant.**

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